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Verminoside- and verbascoside-induced genotoxicity on human lymphocytes: Involvement of PARP-1 and p53 proteins

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ABSTRACT

Verminoside and verbascoside are natural compounds present in plants used in traditional medicine. They exhibit several biological activities including anti-inflammatory, anti-bacterial and anti-tumor properties. The potential applications of these compounds as ingredients in pharmaceutical formulations and cosmetics prompted us to investigate on cytotoxic and genotoxic activity of verminoside and verbascoside on human lymphocytes using genetic toxicity assays recommended in preclinical studies by the US Food and Drug Administration (FDA). We analyzed chromosome aberrations (CAs) and sister chromatid exchanges (SCEs) as well as the mitotic index (MI) and cell viability after the treatments with verminoside and verbascoside. This report is the first to clearly demonstrate a significant increase of structural CAs and SCEs on normal human lymphocytes associated with a reduction of the MI in both verminoside- and verbascoside-treated cells. Moreover, we observed enhanced protein expression levels of PARP-1 and p53 that are key regulatory proteins involved in cell proliferation and DNA repair. Interestingly, mass spectrometric analysis of the compounds in the culture supernatants also showed that verminoside remained unchanged during the culture period while verbascoside was hydrolyzed to its derivative, caffeic acid and the last one seems to be responsible for the observed biological activity.

1. Introduction

Verminoside, an iridoid derivative, and verbascoside, a phenylethanoid compound, are the major constituents of *Kigelia africana* (Lam.) Benth (syn *Kigelia pinnata* DC, Bignoniaceae), a tropical plant used in folk medicine. Remedies prepared from dried fruits are used for emollient, anti-eczema and anti-psoriasis properties. Remedies from root bark are also used for venereal disease treatment and naphtoquinones extracted from *K. africana* also show anti-trypanosomal (Moideen et al., 1999) and anti-microbial (Akunyili et al., 1991) activities and exhibit anti-tumor activity against melanoma and renal carcinoma cells (Houghton et al., 1994; Jackson et al., 2000). Quantitative High Performance Liquid Chromatogrophy (HPLC) analysis of the dried fruit extract from *K. africana* showed that verminoside and verbascoside represented 2.1% and 0.55% (w/w) of the extract respectively (Picerno et al., 2005). Several studies have demonstrated that verbascoside exhibits a number of biological activities including anti-oxidative (Xiong et al., 1996; Wong et al., 2001) anti-bacterial (Rigano et al., 2006) and anti-tumor actions (Kunvari et al., 1999; Ohno et al., 2002; Lee et al., 2007). Verbascoside has been also shown to modulate nitric oxide (NO) production and the expression of inducible nitric oxide synthase (iNOS) in activated macrophages (Xiong et al., 2000; Lee et al., 2005). It also inhibits histamine, arachidonic acid release and prostaglandin E2 production in RBL-2H3 mast cells suggesting a possible application of the compound as anti-inflammatory remedy (J.H. Lee et al., 2006; K.Y. Lee et al., 2006). More recently it has been reported that verbascoside attenuates glutamate-induced neurotoxicity and mitigates scopolamine-induced memory impairment in mice (Koo et al., 2005; J.H. Lee et al., 2006; K.Y. Lee et al., 2006). Despite the expanding literature on verbascoside, very little is known about verminoside. We have previously demonstrated the anti-inflammatory property of verminoside which is able to inhibit iNOS expression and NO release in LPS-induced J774.A1 macrophage cells (Picerno et al., 2005).

Abbreviations: CA, chromosome aberration; FDA, US Food and Drug Administration; ESIMS, electrospray-ionization mass spectrometry; HPLC, High Performance Liquid Chromatogrophy; iNOS, nitric oxide synthase; MI, mitotic index; PARP-1, poly(ADP-ribose) polymerase; PHA, phytohemagglutinin; SCE, sister chromatid exchange.

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On the basis of the overall observations, suggesting the potential applications of verbascoside and verminoside as ingredients of pharmaceutical formulations and cosmetics, here we investigated the cytotoxic and genotoxic activity of both compounds on human lymphocytes. We used chromosome aberration (CA) and sister chromatid exchange (SCE) analyses as genetic end-points and mitotic index (MI) and exclusion of trypan blue dye as markers of cell proliferation and viability. To characterize the observed effects at molecular level we also determined the expression level of poly(ADP-ribose) polymerase (PARP-1) and p53. Indeed, both proteins are required for maintaining genomic integrity (Lane, 1992; Wang et al., 1997), synergize in suppressing chromosomal rearrangements and regulate DNA double strand break repair in primate cells (Kastan et al., 1991; Nelson and Kastan, 1994; Süsse et al., 2004). Finally, we also performed mass spectrometric analysis of verminoside and verbascoside in the culture supernatants in order to assess whether the genotoxic as well as cytotoxic activity of the compounds was due to intact molecules or to their derivatives.

2. Materials and methods

2.1. Chemicals

Verminoside (β-D-glucopyranoside, 6[[3-(3,4-dihydroxyphenyl)-1-oxo-2-propenyl]oxy]-1a,1b,2,5a,6,6a-hexahydro-1a-(hydroxymethyl)oxireno[4,5]cyclopenta[1,2-*c*]pyran-2-yl, [1aS-[1aα,1bβ,2β,5aβ,6β(E),6aα]]) and verbascoside ([2-(3,4-dihydroxyphenylethyl)-1-*O*-α-L-rhamnopyranosyl-(1 → 3)-β-D-(4-*O*-caffeyl)-glucopyranoside]) were extracted and purified as reported by Picerno et al. (2005) (purity of each compound >98%) and dissolved in sterile water. Each compound was added immediately after phytohemagglutinin (PHA) stimulation and left throughout the culture period. Chemical structures of verminoside and verbascoside are shown in Fig. 1.

2.2. Lymphocyte cultures

Blood samples were obtained from three healthy unrelated donors, aged 25–30 years, none of them was receiving drugs or had a history of smoking/drinking. Lymphocytes were separated by Ficoll–Hypaque gradient density. 2×10^6 cells were cultured in 9 ml of RPMI 1640 medium supplemented with 15% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and 10 μg/ml phytohemagglutinin (M form). Cell culture reagents were all obtained from Gibco, Invitrogen. Cells were cultured in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C. Cell survival was determined by the Trypan-blue exclusion method.

2.3. CA, SCE, and MI determinations

Cells were cultured for 72 h at 37 °C for cytogenetic studies. After 70 h of incubation, colcemid (Sigma, 0.2 μg/ml) was added. Lymphocytes were collected by centrifugation, resuspended in prewarmed hypotonic solution (0.075 M KCl) for 20 min, fixed in methanol/acetic acid (3:1) for 10 min, and stained with a 5% Giemsa solution. For SCE analysis, 30 h prior harvesting, BrdU (Sigma) was added to each culture (10 μg/ml) and samples were protected from light. Colcemid was added during the final 2 h of cell growth. Harvested cells were treated with hypotonic solution and fixed with methanol/acetic acid (3:1). Air-dried slides were stained with a 0.2% acridine orange solution in phosphate buffer (pH 6.8) and sealed with paraffin. From each concentrations and from each subject, 50 well-spread metaphases were analyzed for both structural chromosome aberrations and sister chromatid exchanges. Gaps were recorded but not included either in the percentage of aberrant cells or in the aberration frequency. Mitotic index was determined as the percentage of cells (prophases and metaphases) over a total of 1000 nuclei analyzed at random (Lioi et al., 1998).

Data were expressed as mean ± S.E. of three independent experiments. Statistical differences between the treatments and the control were evaluated by one-way analysis of variance (ANOVA). In the case of a significant result in the ANOVA, Student's *t*-test was performed (Lioi et al., 1998; Sarnataro et al., 2006). A *P*-value less than 0.05 was considered statistically significant.

2.4. Western blotting

Mitogen activated lymphocytes were incubated with verminoside (0.05–0.1 mM) and verbascoside (0.05–0.1 mM) for 24 h and 48 h. For protein detection, cells were washed with PBS and resuspended in Tris–HCl 20 mM pH 7.5, 10 mM NaF, 150 mM NaCl, 1% Nonidet P40, 1 mM phenylmethylsulphonylfluoride, 1 mM Na_3VO_4, leupeptin (10 μg/ml), and trypsin inhibitor (10 μg/ml). After 40 min, cell lysates were obtained by centrifugation at 13,000 rpm for 15 min at 4 °C.

Protein concentrations were estimated by the Bio-Rad protein assay using bovine serum albumin as standard. Equal amounts of protein (40 μg) of cell lysates were dissolved in Laemmli's sample buffer, boiled for 5 min, and subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (8% polyacrylamide). Proteins were then transferred to nitrocellulose membrane at 100 mA for 45 min at room temperature.

Filters were blocked with TBS, 5% (w/v) non-fat dry milk for 40 min at room temperature and then skinned overnight at 4 °C with the anti-PARP-1 (Santa Cruz Biotechnology 1:1000) or anti-p53 (Santa Cruz Biotechnology 1:1000) antibody, diluted in TBS, 5% (w/v) non-fat dry milk and 1% tween-20. Blots were then incubated, after four washes in TBS containing 5% (w/v) non-fat dry milk and 1% tween-20, with horseradish peroxidase conjugated goat anti-mouse IgG (1:5000) for 1 h at room temperature. Filter was also incubated in the presence of the antibody against the GAPDH protein.

Immunoreactive bands were visualized using ECL detection system (Amersham, GE Healthcare) according to the manufacturer's instructions and exposed to Kodak X-Omat film. The protein bands of PARP-1 and p53 on X-Omat films were quantified by scanning densitometry (Imaging Densitometer GS-700 Bio-Rad, U.S.A.).

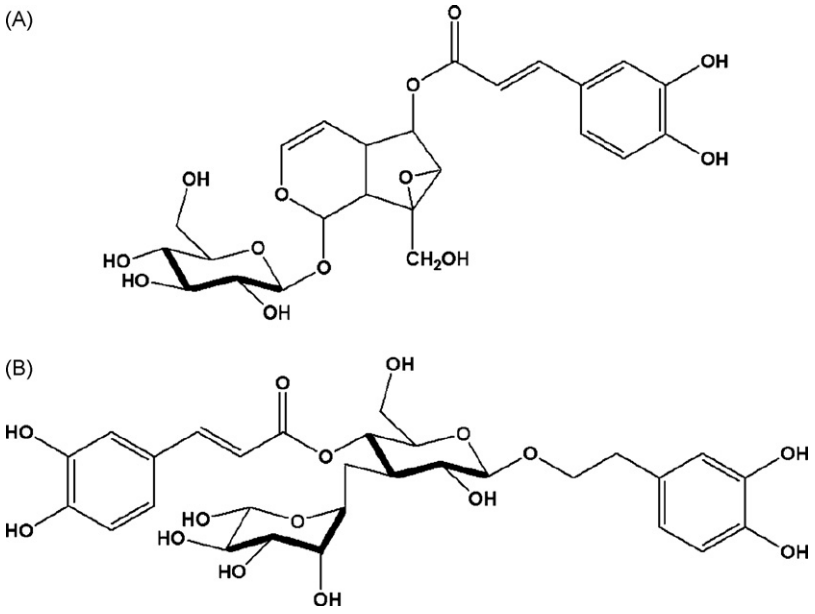

Fig. 1. Chemical structures of verminoside (A), and verbascoside (B).

Data are presented as mean ± S.E. values of three independent determinations. All experiments were done at least three times, each time with three independent observations. Statistical analysis was performed by two-way ANOVA test and multiple comparisons were made by Bonferroni's test. A *P*-value less than 0.05 was considered statistically significant.

2.5. Spectroscopic analysis

Electrospray-ionization mass spectrometry (ESIMS) was recorded using a Finnigan LC-Q Deca instrument (Thermoquest, San Jose, CA) equipped with Xcalibur software. Samples were dissolved in MeOH and infused in the ESI source by using a syringe pump; the flow rate was 3 μl/min. The capillary voltage was set at 5 V, the spray voltage at 5 kV, and the tube lens offset at 35 V. The capillary temperature was 220 °C. Data were acquired in the MS1 scanning mode (*m/z* 150–1500).

ESIMS, in the positive ion mode, of culture supernatants and medium plus verminoside ($C_{24}H_{28}O_{13}$): *m/z* 547 $[M+Na]^+$ corresponding to the molecular formula $C_{24}H_{28}O_{13}Na$; ESIMS, positive ion, of culture supernatants and medium plus verbascoside: *m/z* 203 $[180+Na]^+$ corresponding to the molecular formula of caffeic acid $C_9H_8O_4Na$, *m/z* 159 $[(180–44)+Na]^+$.

3. Results

3.1. Cytotoxicity of verminoside and verbascoside

To determine the cytotoxic activity of verminoside and verbascoside in mitogen-stimulated human lymphocytes, cell viability and mitotic index were determined 72 h after treatment with the compounds. Results showed a concentration-related reduction of cell viability in the concentration range of 0.01–1.0 mM (Fig. 2); this effect was significantly pronounced at 0.05 mM and 1.0 mM in both verminoside- and verbascoside-treated cells (Fig. 2A). At these concentrations we also detected a concentration-dependent inhibition of the mitotic index (Fig. 2B). As a consequence of the reduced cell viability, at 1.0 mM concentration of either verminoside or verbascoside we were unable to analyze mitotic index for both verminoside- and verbascoside-treated lymphocytes. Verbascoside induced a higher ($P < 0.01$) inhibition of cell mitoses at 0.1 mM whereas verminoside seemed to reduce MI in a lesser extent. The parallel decrease of cell survival and mitotic index in both verminoside- and verbascoside-treated lymphocytes, suggests that these compounds negatively affect cell growth and cell viability at the higher concentrations tested; and verbascoside was the most effective.

3.2. Cytogenetic analysis

On the basis of the cytotoxic effects observed, we chose concentrations ranging from 0.01 mM to 0.1 mM for both verminoside and verbascoside in order to obtain comparable results on their genotoxic activity. Table 1 reports the results of the induction of structural chromosome aberrations (chromosome and chromatid breaks and fragments) in verbascoside- or verminoside-treated lymphocytes.

Verbascoside induced a significant and concentration-related enhancement of chromosome aberrations as well as an increase in the percentage of aberrant cells. On the other hand, verminoside induced a significant but concentration-unrelated induction of chromosome aberrations. Notably, multiple aberrations were detected in both verminoside- or verbascoside-treated lymphocytes at all concentrations tested (Table 1).

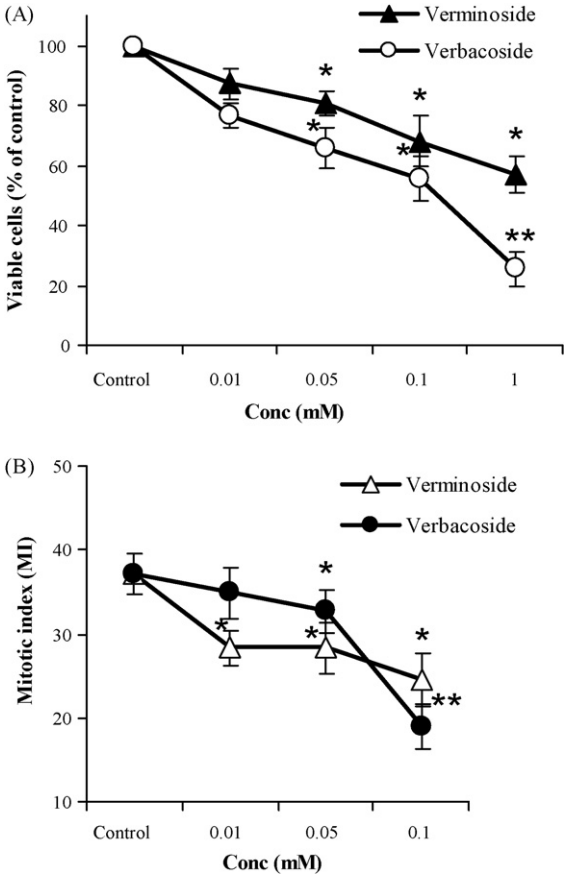

Fig. 2. Cell survival (A), and mitotic index (B) in 72 h verminoside- and verbascoside-treated lymphocytes. Results are given as mean ± S.E. of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ vs control.

Table 1
Chromosome aberrations in human lymphocytes treated with verminoside or verbascoside

Treatment	Concentration (mM)	Chromosome aberrations				Aberration frequency[a] (mean ± S.E.)	Aberrant cells(%)[b] (mean ± S.E.)
		Chromosome breaks	Chromatid breaks	Fragments	Gaps		
Control	–	–	6	–	5	4.0 ± 2.0	4.0 ± 2.0
Verminoside	0.01	3	20	7	14	20.0 ± 4.0*	18.0 ± 2.0*
	0.05	3	26	9	8	25.3 ± 9.4*	20.0 ± 4.0*
	0.1	4	16	7	2	27.0 ± 4.2**	22.0 ± 2.8**
Verbascoside	0.01	3	20	3	4	17.3 ± 1.2*	10.8 ± 3.0*
	0.05	5	23	12	14	26.7 ± 3.1**	20.1 ± 3.1**
	0.1	7	35	13	10	36.7 ± 6.4**	28.7 ± 3.1**

[a] Total number of aberrations without gaps/total number of cells scored × 100.
[b] Total number of cells with at least one chromosome aberration but not gaps/total number of cells analyzed × 100.
* $P < 0.05$.
** $P < 0.01$ vs control.

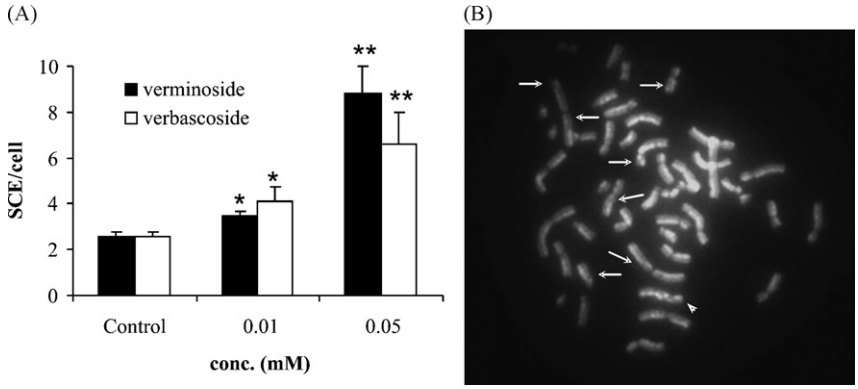

Fig. 3. (A) Number of sister chromatid exchanges (SCE/cell) in 72 h verminoside- and verbascoside-treated lymphocytes. Results are given as mean ± S.E. of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ vs control. (B) Representative metaphase showing a triple sister chromatid exchange (white arrowhead) and other single SCEs (white arrows) following the treatment with verminoside.

Fig. 3 shows the results of SCE analysis carried out on verminoside- or verbascoside-treated lymphocytes in the same donors' group. Both compounds induced a concentration-dependent increase of the number of SCEs/cell compared to control cells. About 3.4 and 2.5-fold induction was reached in human cultured lymphocyte treated with 0.05 mM of verminoside or verbascoside (Fig. 3A). Notably, we also observed an increase of multiple sister chromatid exchanges (double and triple exchanges) in verminoside-treated cells (Fig. 3B).

Since of the cytotoxic activity of these compounds, we did not detect a significant number of second cycle metaphases in lymphocytes treated with 0.1 mM verminoside and verbascoside.

3.3. PARP-1 and p53 expression

PARP-1 and p53 are proteins required for genomic stability and usually activated by agents inducing DNA strand breaks (Wang et al., 1997; Süsse et al., 2004), Since p53 modifications have been found to be related with structural chromosome changes (Agapova et al., 1996), we determined protein expression of PARP-1 and p53 in order to better characterize the mechanism of action of the tested compounds. Our results showed (Fig. 4A) that verminoside significantly increased the expression of PARP-1 in a time- and concentration-dependent manner. In verbascoside-treated lymphocytes we observed a concentration-independent overexpression of PARP-1 after 48 h. Moreover, we did not observe any cleaved form of PARP-1, thus suggesting that the inhibition of cell proliferation and viability did not involve a caspase-dependent apoptotic effect. Western blotting analysis of p53 expression (Fig. 4B) also showed that both verminoside or verbascoside significantly increased p53 in a concentration-related manner only after 48 h of treatment, while a general decrease of p53 expression was detected in mitogen-stimulated human lymphocytes untreated with the test substances (controls). At 72 h treatment, we did not detect any further variation of both PARP-1 and p53 (data not shown).

3.4. Mass spectrometry analyses of verminoside and verbascoside in culture supernatants

Electrospray-ionization mass spectrometry of culture supernatants and medium plus verminoside or verbascoside was performed after incubation at 37 °C for 72 h in order to investigate the fate of the compounds during cell treatment. Results showed that verminoside was not degraded during the treatment. The ESIMS, in positive mode, of the medium and supernatant of the culture media of lymphocytes treated with verminoside showed the molecular ion peak of the intact compound at m/z 547 $[M+Na]^+$, and MS fragmentation pattern superimposable to that of the parent compound and consistent with that previously reported for untreated verminoside (Picerno et al., 2005). On the contrary, verbascoside was completely cleaved either in presence and absence of cells; in fact, the ESIMS, in positive ion mode, of supernatant of the culture media of lymphocytes treated with verbascoside as well as of the medium showed fragments at m/z 203 $[180+Na]^+$ and m/z 159 $[(180–44)+Na]^+$, ascribable to caffeic acid unit and the loss of a carboxylic group (44 mass unit) from caffeic acid, respectively. No molecular ion peak of intact verbascoside (m/z 625) was observed in the MS spectra. These findings suggest that the biological effects we observed, in our experimental conditions, were due to the action of verminoside but were due to caffeic acid moiety in the case of verbascoside-treated cells.

4. Discussion

Verminoside and verbascoside are natural compounds extracted from air-dried fruits of *K. africana* (Picerno et al., 2005). Particularly, verbascoside is a phenylethanoid glycoside widely distributed in many other plants used in traditional medicine (Pu et al., 2003). Recent reports have underlined that verminoside and verbascoside show some interesting biological activities including anti-inflammatory and anti-cancer properties (Picerno et al., 2005; J.H. Lee et al., 2006; K.Y. Lee et al., 2006; Lee et al., 2007). As a part of a safety evaluation of novel ingredients to be used in pharmacological applications, the cytotoxic and genotoxic activity of verminoside and verbascoside were examined on human lymphocytes. The treatment of cells with verminoside or verbascoside, used at comparable concentrations, significantly reduced cell proliferation and enhanced cell mortality particularly in verbascoside-treated lymphocytes. On the basis of the observed reducing trends of the mitotic index and cell viability and as suggested by the analysis of cell recovery in human lymphocytes treated with verbascoside until 72 h (data not shown), it is possible to speculate that the cytotoxic activity of the compound could be ascribable in part to a delay/arrest in cell cycle progression. These results corroborate our previous data concerning the cytotoxic capability of verbascoside in LPS-induced J774.A1 macrophages (Picerno et al., 2005) and are in line with those obtained by Lee et al. (2007) who evidenced that verbascoside inhibited human promyelocytic HL-60 cell proliferation. Cytogenetic analysis performed by using CA and SCE tests indicated a significant increase of structural CAs and SCE frequencies in both verminoside- or verbascoside-treated lymphocytes, even though SCE curves were quite flat. Indeed, it has been reported that a number of agents creating strand breaks, includ-

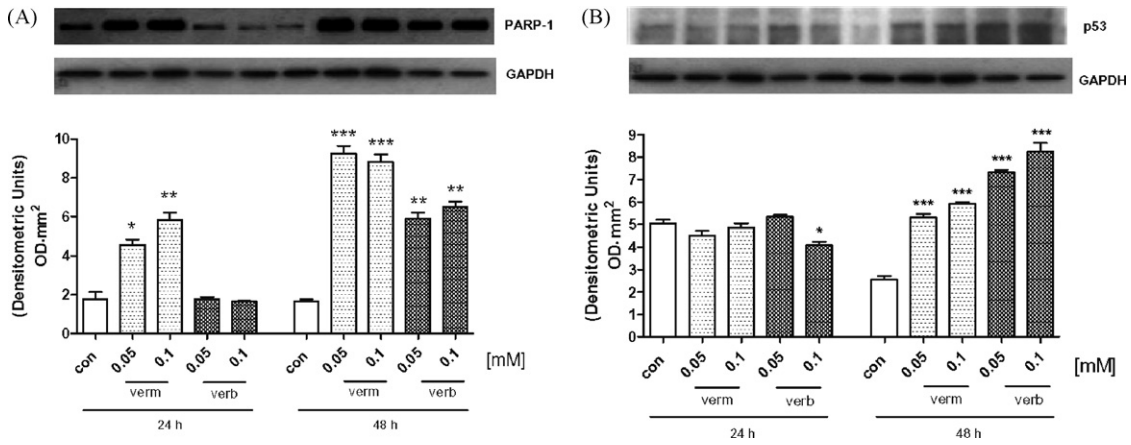

Fig. 4. Protein expression and densitometric analyses of PARP-1 (A), and p53 (B). GAPDH was used as an internal control. Western analysis was performed three times and the most representative blot is shown. Values, mean ± S.E., have been calculated from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs control.

ing ionizing radiation and restriction enzymes are poor inducers of SCEs (Perry and Evans, 1975; Morgan et al., 1988). Moreover, it has been demonstrated that as a consequence of the treatment with DNA damaging agents, different DNA repair pathways, leading or not to detectable SCEs, could be activated (Johnson and Jasin, 2000).

In order to gain further insights into the mechanisms of action of verminoside- or verbascoside-induced genotoxicity, expression of PARP-1 and p53 proteins, both required for genomic stability (Kastan et al., 1991; Nelson and Kastan, 1994; Süsse et al., 2004) were analyzed in verminoside- or verbascoside-treated lymphocytes obtained from the same donors' group. Our results indicate that the expression of PARP-1 was significantly increased after verminoside treatment in a time- and concentration-dependent manner, whereas in verbascoside-treated lymphocytes, the peak of overexpression of PARP-1 was reached only 48 h after the treatment, independently from the concentration. Analysis of the expression of p53 indicated that both verminoside and verbascoside significantly increased p53 in a concentration-related way only after 48 h of treatment. All these observations suggest that, as a consequence of the genotoxic insult produced by verminoside or verbascoside, cells activate DNA repair events enhancing the expression of PARP-1 and p53 in order to repair DNA damage. Interestingly, p53 expression decreased in controls during the culture period with a peak at 48 h. It is possible that the treatment with PHA, necessary for culturing cells until 72 h and to induce mitotic division, could be responsible for the decrease of p53 expression. Indeed, the tumor suppressor p53 is a key protein required for both cellular response to DNA damage and maintenance of G1 cell cycle phase in differentiated cells (Vogelstein et al., 2000). Moreover, it has been reported that p53 gene product is a negative regulator of mitotic cyclin B1 in normal cells (Innocente et al., 1999). Süsse et al. (2004) demonstrated that PARP-1 synergizes with p53 in suppressing chromosomal rearrangements and it has been proposed that PARP-1 acts upstream of p53 in the damage signaling pathway directly, via physical interactions with poly(ADP-rybosyl)ation of p53 or, indirectly, via activation of the DNA strand break sensing ATM kinase which phosphorylates p53 (Whitacre et al., 1995; Vaziri et al., 1997; Valenzuela et al., 2002; Süsse et al., 2004). Our data indicating that verminoside- or verbascoside-treated cells try to repair DNA damage enhancing the expression of PARP-1 and p53 are in line with these findings. We hypothesize that the continuous exposure to these substances until 48 h resulted in the ability of treated cells to increase PARP-1 and p53 protein expression not enough to maintain genome integrity. It is probably that cell death does not occur via a caspase-dependent apoptotic pathway because no cleaved form of PARP-1 was observed. Moreover, it is possible to speculate that the overexpression of PARP-1 that we detected could be responsible for the small, albeit significant, induction of SCEs, in fact Wang et al. (1997) demonstrated that cells with non-functional PARP-1 show increased levels of SCEs following genotoxic treatment with Mytomicin C.

We performed cytogenetic analysis on 72 h cultured lymphocytes in order to provide evidences on the effects of a prolonged exposure to the compounds. However, this incubation time is not generally considered optimal to detect structural chromosome aberrations, whose frequency may be underestimated as a consequence of second mitoses, but this does not affect the conclusion regarding the significance of genotoxicity of both verminoside and verbascoside.

Finally, the mass spectrum determinations of verminoside or verbascoside in the cell medium, revealed that verminoside remained intact for the whole incubation period while verbascoside was cleaved in its constituent, caffeic acid. This finding indicates that verminoside acts on cells as intact molecule, while verbascoside-induced DNA damage, PARP-1 and p53 expression through caffeic acid.

In summary, our study provides the first evidence of genotoxicity of verminoside and verbascoside on human lymphocytes using genetic toxicity tests recommended by Food and Drug Administration (FDA) for risk assessment. Our data provides evidences that both compounds are genotoxic even though treated cells activate PARP-1 and p53, two key proteins required in cell cycle progression and DNA repair. Since verminoside and verbascoside exhibit various biological activities and they could be used as ingredients in pharmaceutical formulations, the data here presented can contribute to risk/benefit assessment and to set the threshold of safe levels.

Conflict of interest

All authors disclose any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the work submitted that could inappropriately influence the present work.

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